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Radioimmunoassay of 17α-Alkylated Anabolic Steroids

By *R. Hampl*,

Research Institute of Endocrinology, Prague, Czechoslovakia,

J. Pícha,

Research Institute of Animal Production, Prague-Uhříněves,

B. Chundela,

Laboratory of Doping Control, Prague and

L. Stárka

Research Institute of Endocrinology, Prague, Czechoslovakia

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Summary: A method for the detection of anabolic 17α-alkylated androstane derivatives in both plasma and urine is described and evaluated. The goat and rabbit antisera against 17α-Methyltestosteron-3-carboxymethyloxim-Rinder-serum albumin were raised and compared using [^{3}H]methandrostenolone as a tracer. 22 Steroids including 10 potent synthetic anabolics were tested for their cross-reaction with these antisera.

Radioimmunoassay für 17α-alkylierte anabole Steroide

Zusammenfassung: Ein selektiver Radioimmunoassay für anabole 17α-alkylierte Androstanderivate im Plasma und im Harn wird beschrieben und ausgewertet. Antiserum gegen 17α-Methyltestosteron-3-carboxymethyloxim-Rinder-serumalbumin wurde an Ziegen und Kaninchen gewonnen. Beide Antisera wurden verglichen unter Anwendung von [^{3}H]Methandrostenolon als Radioligand. Die Kreuzreaktion wurde für 22 Steroide, davon 10 wirksame Anabolika, ermittelt.

Introduction

In spite of widespread use of anabolic steroids in human and veterinary medicine, a method of quantification of these drugs in body fluids has been lacking for a long time. Only few papers have dealt with their distribution and kinetics in the organism, probably due to the limited number of respective radioactive steroids available (1, 2). Nowadays, the problem of detection of anabolic steroids has become urgent with respect to their abuse in sports.

A selective and sufficiently sensitive radioimmunoassay seemed to meet these requirements and, indeed, a method has been developed recently in *Brooks*' laboratory (3, 4), permitting the detection of several 17α-alkylated androstane derivatives and 19-norsteroids. The preparation of rabbit antisera agains steroid 3-carboxy-methyloxime-bovine serum albumin conjugates was reported (3, 4), but the detailed data on their specifity were not given. [^{125}I]tyraminyl derivatives of steroids were used as tracers.

In the present paper, a radioimmunoassay for an important and most frequently used class of perorally active anabolic steroids, i. e. of those alkylated at the 17α-position is described, using antisera raised against identical immunogen as reported by *Brooks* (3) and

with [^{3}H]methandrostenolone as a tracer. The antisera were raised in both goats and rabbits and tested for specifity.

Materials and Methods

Materials

[1,2,4(n)-^{3}H]methandrostenolone (17α-methyl-17β-hydroxy-1,4-androstadien-3-one, methandienone), specific radioactivity 1591 GBq · mmol^{-1} from Radiochemical Centre, Amersham, England was purified by paper chromatography in the system cyclohexane toluene methanol water 9 : 1 : 8 : 2 (volumes, 9 ml + 1 ml + 8 ml + 2 ml). Its radiochemical purity exceeded 97%. Methyltestosterone-3-carboxymethyloxime was prepared according to *Erlanger* et al. (5). Its identity was confirmed by physicochemical data. Its melting point was 179°C. The ultraviolet spectrum in 0.05 mol · l^{-1} Tris-HCl buffer, pH 8.3 (Unicam Model SP 800 spectrophotometer) showed λ_{max}254 nm with ϵ_{254} 18 200 cm^{2}mol^{-1} and infrared spectrum (Perkin Elmer Model 254 spectrometer, KBr pellet) gave ν_{max} = 3440 cm^{-1} (hydroxyl), 1740 cm^{-1} (C = 0 of carboxyl), no band in the region 1680–1640 cm^{-1} (conjugated C = 0). Methyltestosterone-3-carboxymethyloxime-bovine serum albumin was prepared by the mixed anhydride method (6), the starting steroid: bovine serum albumin molar ratio was 45. Following repeated precipitation with acetone at pH 4.5 and dialysis, the product was freeze-dried and the protein content in the lyophilized material was determined according to *Lowry* et al. (7), with the original bovine serum albumin as a standard. The number of NH_2-residues substituted by steroid assessed by ultraviolet absorption at 254 nm was 19 per protein molecule. Anabolic steroids, methandienone (Stenolon), dimethylandrostanolone (1α, 17α-dimethyl-17β-hydroxy-5α-androstan-3-one, Demalon) and 19-nortestosterone phenylpropionate (Superanabolon) were obtained from Spofa (Czechoslovakia); stanazolol (17α-methyl-17β-hydroxy-5α-androstane-3,2-C-pyrazol, Stromba) was purchased from CIBA (Switzerland); other anabolic steroids were obtained from Schering A.G. (Berlin). All other non-radioactive steroids were purchased from Koch and Light (England). Chemicals were of analytical grade, solvents were twice distilled before use. Only silanized glassware was used for radioimmunoassay.

Methods

Immunization

Immunogen methyltestosterone-3-carboxymethyloxime-bovine serum albumin (1 mg), suspended in equal volumes of 90 g · l^{-1} saline and complete *Freund* adjuvans (1 ml of the mixture) was injected subcutaneously into three New Zealand white rabbits (1 mg of conjugate per animal) or three goats (3 mg per goat), biweekly for 6 weeks and every month thereafter. Blood for titer estimation was withdrawn from the central ear vein in rabbits or the jugular vein in goats. The highest titer was achieved between the 2nd and 3rd week following a booster. The animals were then killed, bled out, and the sera divided into 10 ml portions and lyophilized. No loss of the binding activities was observed during 6 months when stored at – 15°C.

Sample preparation

Plasma (250 µl) or urine (5 ml) was extracted with diethyl ether (3 ml or 10 ml, respectively), the organic phase washed with 90 g · l^{-1} saline (1 ml), the aqueous layer was frozen and the organic layer was decanted into reaction tubes. The extraction tubes were rinsed with ether (1 ml), which was added to the main portion, and the solvent was evaporated. 100 µl each of anhydrous pyridine and acetic anhydride, were added to the dry residue, agitated on Vortex mixer and left at 37°C for two hours. The reagent was then evaporated to dryness under nitrogen. Simultaneously, the dry residues of standards were worked up in the same way.

Radioimmunoassay

To the dry residues of samples, or increasing amounts of steroid for the calibration curve, were added 100 µl each of [^{3}H]methandrostenolone (333 Bq) and appropriately diluted (usually 1 : 4000) antiserum, and the volume was adjusted to 0.5 ml with buffer. 0.1 mol · l^{-1} sodium phosphate, pH 7.2 containing 90 g · l^{-1} saline, 1.0 g · l^{-1} sodium azide and 1.0 g · l^{-1} gelatine was used. The mixture was shaked on a Vortex mixer, incubated at 37°C with constant shaking for 30 min, then left at 4°C for 1 hour. Cold, stirred suspension of dextran-coated charcoal (0.5 ml of 2.5 g · l^{-1} Norit A and 0.25 g · l^{-1} Dextran 70) was then added. The samples were shaken again on a Vortex mixer. After 10 min standing at 4°C they were centrifuged for 10 min, and aliquots (0.5 ml) of supernatant were removed for the determination of radioactivity.

Radioactivity measurement

Radioactivity was measured on a Betaszint BF 5000 liquid scintillation spectrometer (Berthold and Frieseke, Federal Republic of Germany), using the external standard channel ratio method for disintegration computing of ^{3}H, with an efficiency of approx. 45%. The scintillation fluid consisted of 4 g PPO, 50 mg of POPOP and 20 ml of methanol in 1 liter of toluene.

Results

Antisera from goats and rabbits were compared with respect to their titer and specifity.

Titer, defined as final dilution of serum at which more than 50% of total radioactivity in the system remained in the supernatant, was highest in one of the goats (1 : 8000). The highest titer achieved in rabbits was 1 : 6000. The titer of antisera was not changed by lyophilization and storage at – 15°C for 6 months.

Specifity of both antisera was evaluated as ability of steroids to cross-react with antibodies. Naturally occurring hormonal steroids, their acetates and commonly used synthetic anabolics were tested. From the percentage of radioactivity remaining in the supernatant in the presence of the competitor and respective amounts of the steroids, the log-logit straight lines were constructed (8), each from at least five points. The amounts of steroids required for 50% displacement of tracer were determined. The displacing abilities of 22 steroids related to methandienone are shown in table 1.

The reliability criteria of the method have been evaluated as demonstrated below, using high titer goat antiserum in a final dilution of 1 : 4000.

Calibration curves, prepared with untreated and acetylated methandienone are shown in figure 1. As may be seen, the acetylation influences the slope of the curve. The reason of this effect is not quite clear, since methandienone itself is not acetylated under the conditions used. Therefore, only the latter curve may be used for analysis of anabolics in biological materials, where the acetylation step is involved. The presence of residual diethyl ether had no effect on the curve.

Recovery of radioactive tracer (167 Bq of [^{3}H]methandienone per sample) following extraction and acetylation averaged 85.9% for plasma and 79.9% for urine.

Tab. 1. Ability of various steroids to bind with rabbit and goat methyltestosterone antibodies as percentage of cross reaction.

Steroid (Systematic name)	Cross reaction in % Rabbit	Goat
Synthetic anabolics – 17α-alkylated		
methandienone (17α-methyl-17β-hydroxy-1,4-androstandien-3-one)	100	100
methyltestosterone (17α-methyl-17β-hydroxy-4-androsten-3-one)	132	163
dimethylandrostanolone (1α, 17α-dimethyl-17β-hydroxy-5α-androstan-3-one)	16.6	39.5
methylandrostendiol (17α-methyl-5-androstene-3β, 17β-diol)	20.2	39.0
stanazolol (17α-methyl-17β-hydroxy-5α-androstane-3, 2-C-pyrazol)	5.9	7.2
fluoxymestrone (17α-methyl-9α-fluoro-11β, 17β-dihydroxy-4-androsten-3-one)	4.7	6.5
oxymetholone (2-(hydroxy-methylene)-17α-methyl-17β-hydroxy-5α-androstan-3-one)	3.2	9.6
norethandrolone (17α-ethyl-17β-hydroxy-19-nor-4-androsten-3-one)	2.3	5.5
Synthetic anabolics – not 17α-alkylated		
nortestosterone (17β-hydroxy-19-nor-4-androsten-3-one)	2.9	1.9
nortestosterone phenylpropionate	< 0.01	< 0.01
methenolone (1-methyl-17β-hydroxy-5α-androst-1-en-3-one)	0.05	0.09
drostanolone (2α-methyl-17β-hydroxy-5α-androstan-3-one)	0.08	0.35
Androgens and their derivatives		
testosterone	47.6	19.8
testosterone acetate (17β-acetoxy-4-androsten-3-one)	0.01	0.02
dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)	6.9	9.7
dihydrotestosterone acetate	0.01	0.01
androstenedione (4-androstene-3, 17-dione)	1.0	0.09
dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one)	< 0.01	< 0.01
androsterone (3α-hydroxy-5α-androstan-17-one)	< 0.01	< 0.01
Other hormonal steroids		
estradiol	0.06	0.03
cortisol	0.01	0.02
progesterone	0.06	0.13

Sensitivity and precision

Sensitivity of the method was expressed as the least amount of non-radioactive methandienone which, when added to normal urine or plasma, caused a statistically significant decrease of measured supernatant radioactivity at the 99% level. As shown in table 2, 100 pg (plasma) and 200 pg (urine), respectively, met these criteria. The respective coefficients of variation are also shown in table 2. An average variation coefficient for concentrations of methandienone added to plasma or urine was 4.95% and 4.60%, respectively.

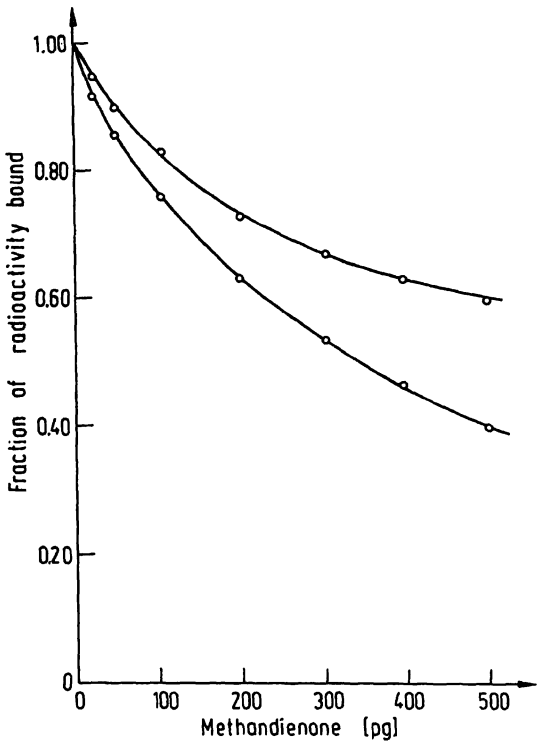

Fig. 1. Effect of acetylation of methandienone on the standard curve prepared with goat anti-methyltestosterone-3-carboxymethyl-oxime-bovine serum albumin. (Each point represents the mean of five determinations).
upper curve = acetylated
lower curve = non-acetylated standard

Accuracy

From the data presented in Table 2, the average recovery of methandienone added to plasma or urine were obtained in the usual way, using "acetylated" calibration curve. The results are summarized in table 3.

Discussion

The aim of this study was to develop a simple and rapid radioimmunoassay method for anabolic steroids suitable as a screening test in a doping control, and to establish its limitations due to the specificity of antisera. It may, however, be used in both human and veterinary medicine in studies on the secretion and rate of metabolism of various anabolic steroids.

The same immunogen as prepared by *Brooks* et al. (3,4) was applied herein and characterized in greater detail. Tritiated steroid was used as a tracer. The antisera from rabbits and goats were elicited; the latter appeared to have a higher titer and bound 17α-methylsteroids more selectively. Since the sensitivity of detection of methyl-

Tab. 2. Sensitivity and precision of methandienone radioimmunoassay in plasma and urine. Increasing amounts od methandienone were added to samples of normal male plasma or urine which were processed as described in methods. Eight determinations were carried out in each group. The differences in radioactivities between the first (i.e. without steroid added) and following groups were evaluated statistically.

Methandienone added	Supernatant radioactivity					
	Plasma			Urine		
	mean ± S.D.	coefficient of variation	significance	mean ± S.D.	coefficient of variation	significance
pg	Bq	%		Bq	%	
none	34.15 ± 1.95	5.71	–	23.95 ± 1.02	4.24	–
50	32.35 ± 1.57	4.84	not signif.	23.50 ± 1.03	4.39	not signif.
100	29.28 ± 1.45	4.95	$p < 0.01$	22.5 ± 0.98	4.37	$p < 0.05$
200	27.60 ± 1.15	4.17	$p < 0.01$	20.68 ± 0.82	3.95	$p < 0.01$
300	26.42 ± 1.25	4.73	$p < 0.01$	17.68 ± 1.17	6.60	$p < 0.01$
500	22.00 ± 1.17	5.30	$p < 0.01$	17.40 ± 0.70	4.02	$p < 0.01$

Tab. 3. Accuracy of methandienone radioimmunoassay in plasma and urine. Recovery of methandienone added to samples of normal male plasma and urine

Methandienone added	found		percentage recovery	
	plasma	urine	plasma	urine
pg	pg	pg	%	%
none	19	86	–	–
100	139	157	120	71.0
200	170	218	75.5	66.0
300	220	456	67.0	123.3
500	542	530	104.6	88.8
		mean:	91.8	87.3

testosterone and closely related methandienone approaches the value 100 pg, nanogram amounts of those steroids with a cross-reaction higher than 5% may still be detected in both plasma and urine. In spite of considerable cross-reaction of testosterone and dihydrotestosterone, these endogenous hormones may be completely eliminated by acetylation (3, 4), since their acetates do not react with the antibody. Under the conditions used, 17α-alkylated 17β-hydroxysteroids do not form 17β-acetates (3); therefore most of 17α-methylderivatives of testosterone and dihydrotestosterone may be detected (see tab. 1). The exception is oxymetholone which forms a 2-acetylderivative. On the other hand, it is possible to estimate norethandrolone, the 17α-ethylsteroid which is also the only circulating and main metabolic product of ethylestrenol (17α-ethyl-17β-hydroxy-19-nor-4-androstene). The cross-reaction of other hormonal steroids and their principal metabolites, especially 17-oxosteroids, is unsubstantial.

The method was developed especially for use as a rapid screening test in doping control. The control of doping depends practically only on analyses of urine collected after the performance. To obtain rapid information, only free steroids are commonly determined. The influence of reduced and hydroxylated metabolites in the conjugated form, either from exogenous or endogenous origin, can be neglected here.

The reliability criteria of the method seem to be sufficient and, with regard to the fact that a large group of compounds is involved, comparable with common radioimmunoassay procedures.

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Dr. R. Hampl
Research Institute of Endocrinology
Národní 8
ČS-116 94 Praha 1, Czechoslovakia